United States Department of Agriculture
Animal and Plant Health Inspection Service
Plant Protection and Quarantine

Frequently Asked Questions
Phytophthora ramorum Diagnostics

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1) What is ELISA?

Enzyme-linked immunosorbent assay (ELISA) is able to detect proteins that belong to specific microbes and viruses. ELISA has been used for medical, veterinary, and plant disease diagnostics for many years to detect pathogens such as HIV, anthrax, small pox, and plum pox virus. ELISA works by having specific antibodies that can recognize proteins that are unique to specific organisms. The specificity of the ELISA is a function of the ability of the developer of the diagnostic to identify a protein that is unique to a particular organism. Please note that this test is not specific for the microbe that causes Sudden Oak Death (*Phytophthora ramorum*), but can detect several species of *Phytophthora*. However, it is useful as a first screen to remove samples that are not positive early on.

For additional technical information on ELISA, please see:

- http://www.biology.arizona.edu/immunology/activities/elisa/technique.html
- http://www.agdia.com/cgi_bin/catalog.cgi/92600

2) What is PCR?

The Polymerase Chain Reaction (PCR) can rapidly detect DNA that is unique to an organism. PCR can be highly sensitive, rapidly detecting a limited number of specific DNA molecules. Other advantages of PCR are that the process occurs in a single tube, and the reaction can often proceed in solutions that may have other contaminants present. It is far more sensitive than ELISA.

The PCR assay is the basis for many other DNA detection assays including Reverse Transcriptase PCR (RT-PCR) for detection of RNA molecules, nested PCR, and Real-Time PCR, and others. **At the present time the nested PCR assay is the only USDA APHIS PPQ validated method for PCR detection of this organism.**

A good tutorial can be viewed on the web at: http://www.lsic.ucla.edu/ls3/tutorials/gene_cloning.html (requires Flash Player or QuickTime). Other animations can be found at:

- http://www.dnalc.org/shockwave/pcranwhole.html
- http://allserv.rug.ac.be/~avierstr/principles/pcrani.html
- http://academy.d20.co.edu/kadets/lundberg/animations.html

- http://faculty.plattsburgh.edu/donald.slish/PCRmov.html

The various methods used for detection of *Phytophthora ramorum* are described, below.

- Nested PCR

Nested PCR is nothing more than a repeat of the PCR reaction with conditions that amplify a smaller fragment than the first reaction. i.e., the DNA target in a nested reaction is always found within the target amplified in the first reaction, and thus the product of the nested PCR is always smaller than the product of the first step.

Nested PCR is used for two main reasons: 1) to increase the specificity of the PCR. In this case, the nested PCR can be used to distinguish between a false positive and a true positive, since only a true positive will amplify products of the correct sequence internal to the first PCR product. 2) Nested PCR is more sensitive. **Nested PCR is the most sensitive method known for detection of *P. ramorum*, and is currently the only APHIS-PPQ validated method for PCR detection of this organism.**

We currently do not know of an available graphic representation of nested PCR, but there is a description at: http://www.bio.davidson.edu/courses/genomics/method/NestedPCR.html However, if you follow the tutorial for regular PCR, you can imagine that running a second PCR after the first one could be done if you use primers that make a product within the primary target sequence.

- Multiplex PCR

Multiplex PCR is simply a PCR reaction where two or more targets are detected in the same reaction. This method is less time-consuming than nested PCR, and can be very useful when one wishes to include an internal control that will indicate that the reaction is working properly. For example, Oregon State University uses a multiplex PCR in which one pair of primers amplifies DNA for the Sudden Oak Death microbe while the other pair amplifies plant DNA always found in the sample. This tells the scientists that the DNA extraction and the PCR itself are working well. APHIS-PPQ will use this test, at times, in conjunction with nested PCR, but it is not used routinely, as it is less sensitive than nested PCR.

- RT-PCR

Reverse transcription PCR (RT-PCR) is typically used for the detection of many plant viruses because they don't have DNA, but rather have RNA. In this case, the PCR is preceded by a step that uses an enzyme (called Reverse Transcriptase) to convert the RNA target to DNA. Otherwise, it is identical to PCR.

See:

- http://www.bio.davidson.edu/courses/genomics/RTPCR/RT_PCR.html
- http://www.csl.gov.uk/science/organ/environ/bee/rnd/virusdetection.cfm

- Real-Time PCR

Real-Time PCR is a variation of PCR that combines the amplification ability of PCR with a fluorescent detection system for analyzing results as the products are made (hence Real-Time). The fluorescent label significantly enhances our ability to detect the product(s) of PCR, and thus increases the sensitivity of the reaction. Another advantage of using Real-Time PCR is that it is suitable for high throughput capability. (However, instrumentation for Real-Time PCR can be expensive, from $40,000 to $95,000+, and development of methods can require greater expertise than may be available to some laboratories.) Depending on the Real-Time PCR method used, the fluorescent label is also a DNA probe in itself, thus adding to the specificity of the reaction.

For information Real-Time PCR, see:
http://www.vetscite.org/issue1/tools/txt_leut_0800.htm or
http://www.vetscite.org/issue1/tools/leute_2_0800.htm
for a good description and animation of Real-Time PCR.

The web site, http://ccm.ucdavis.edu/cpl/Tech%20updates/TechUpdates.htm, has some very good descriptions of these procedures. (For a description of Real-Time PCR, see the section on "Fluorogenic 5' Nuclease PCR (Real Time PCR)".

3) **The terms 'culture negative' and 'morphology negative' have been used to describe results of some culturing tests. What do these mean?**
A "culture negative" means that nothing grew on the PARP media. "Morphology negative" means that a *Phytophthora* sp. other than *P. ramorum* was isolated.

4) **What does it mean if I have a positive PCR result and a negative culture result?**
It is possible to obtain a nested PCR positive result in a situation where the DNA of *P. ramorum* is present, but the organism is no longer viable, however we are not aware of this having ever been observed. We are not aware of example where this has occurred. A more likely explanation for a positive PCR result combined with a culture negative is that the organism is dormant because the sample was collected at a time when the conditions were not conducive for active growth of the organism. A third explanation is that the plant had been treated with a fungicide that is preventing the growth of the organism from the plant tissue. Holding the plant and re-sampling should allow the pathogen to be isolated, if the effects of the fungicide are reduced or eliminated, and if the conditions are suitable for pathogen growth.

There is anecdotal evidence that isolation of *P. ramorum* from some hosts is easier than from others. This is likely the case, but that does not mean that the organism is easily isolated from 'easy' hosts all the time or from every cultivar. There is still much about the biology of this organism which is not known. APHIS-PPQ strongly recommends that multiple attempts be made to isolate the organism from any plant(s) that tested positive using the nested PCR. There is evidence that repeated isolation attempts are likely to result in isolation of the pathogen.

It is also possible to obtain a false negative result (meaning the diagnostic test indicates no pathogen detected, when, in fact, it is present) in the case of a "morphology negative" result with a PCR positive result, but the frequency with which this may occur is not known. There is anecdotal evidence at this time that other *Phytophthora* spp. may outgrow or out-compete *P. ramorum*. In this case the initial negative result could be considered preliminary while the lab continues to try to isolate *P. ramorum*.

5) **Is it possible to obtain a false positive result (meaning that the diagnostic used incorrectly indicated that the pathogen was present when, in fact, it is not) using the PPQ validated nested PCR?**
We are not aware of an example where this has occurred when the PPQ validated nested PCR assay was followed exactly.

6) **What about the other PCR methods being used for diagnosis of SOD?**
Currently, only the nested PCR, developed at the University of California Berkeley (http://www.aphis.usda.gov/ppq/ispm/sod/survey.html) has been validated by PPQ-CPHST, and it is only results of this assay, performed at the NPGBL or some other laboratory specifically designated by PPQ, that will be accepted by PPQ for regulatory purposes.

The "multiplex" PCR assay, developed at Oregon State University, is a useful assay, since it does provide some information on the quality of the sample DNA. It is not, however, as specific as the nested PCR method, but is useful for routine screening and as a second test to determine that DNA was in fact present in the nested PCR.

Some laboratories have performed PCR-based diagnostics for *P. ramorum* using only the 1st stage of the nested PCR. We do not recommend this, as it is considerably less sensitive than the complete nested PCR, potentially leading to false negative results. DNA can be amplified non-specifically in such a way that the result can be misinterpreted as a positive.

7) **Why use ELISA as a primary screening tool?**
We recommend using ELISA as a preliminary screening tool for the presence of Phytophthora in order to pinpoint which plants need to be plated onto PARP for isolation attempts using procedures described elsewhere (WWW). If a suspect Phytophthora ramorum is isolated, this culture would be forwarded to PPQ/NIS where it a determination of morphology positive or negative would be made. DNA would isolate from the culture by NPGBL for PCR testing to determine whether the culture is PCR positive or negative.

8) **Does it ever happen that the organism is cultured, but the PCR is negative for plant tissue?**
Yes, but to the best of our knowledge, this is rare. It is possible for this to happen in cases where leaf (or stem or twig) tissue was sampled for DNA and isolation attempts

were made from a companion sample (from the same leaf, for example). It may be that the organism simply was not present in the plant material used for DNA sample or was present in such a low concentration that there was insufficient DNA for the successful PCR. There are also inhibitors that occur in plants that can be carried through the DNA purification process that may affect PCR. Using the PPQ-validated method should help avoid this, but we do not have data on inhibitors for every possible host of *P. ramorum.* The current recommended procedure involves isolating DNA from cultures of putative *P. ramorum*.

In this situation the culture would be tested using the validated PCR assay. Additionally, one or several additional plant samples could be tested using the validated PCR assay.

Abbreviations:

- APHIS – Animal and Plant Health Inspection Service (an agency of USDA)
- CPHST – Center for Plant Health, Science and Technology (part of USDA-APHIS-PPQ)
- DNA – Deoxyribose Nucleic Acid
- ELISA – Enzyme-linked immunosorbent assay
- NIS – National Identification Service (part of USDA-APHIS-PPQ)
- NPGBL – National Plant Germplasm and Biotechnology Laboratory (part of USDA-APHIS-PPQ)
- PARP - pimaricin-ampicillin-rifampicin-PCNB agar, a media selective for Phytophthora species
- PCR – Polymerase Chain Reaction
- PPQ – Plant Protection and Quarantine (Part of USDA-APHIS)
- RNA – Ribose Nucleic Acid
- USDA – United States Department of Agriculture
- WWW – World Wide Web